Prevalence of Extended-Spectrum β-Lactamases (ESBLs) and AmpC β-Lactamases in Clinical Isolates of Multiple Drug-Resistant Escherichia coli

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Abstract

The current study aims to investigate the prevalence of extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases in multiple-drug-resistant Escherichia coli (e.g., multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR)) collected from hospitalized patients in Baghdad. The results showed that the prevalence of ESBLs among E.coli isolates was high. From the total 113 E.coli isolates, 75 (66.37%) were ESBL-producing, while 38 (33.63%) were non-ESBL-producing. Out of 75 ESBL-positive isolates, 39 (52%) were obtained from females and 36 (48%) from males. Additionally, 43 (57.33%) isolates were collected from urine samples, and the highest production of ESBLs was obtained from the age group 41–60 years (29.33%). Moreover, out of 111 MDR E.coli, 64 (57.66%) exhibited a positive ESBL test, while 47 (42.34%) did not. Out of 24 XDR E.coli isolates, 11 (45.83%) demonstrated positive ESBLs, while 13 (54.17%) showed negative ESBLs. The antimicrobial susceptibility test results showed that positive ESBL E.coli isolates were more drug-resistant than negative ESBL isolates. The positive ESBLs of E.coli exhibited a higher resistance rate to the β-lactam antibiotics and showed a co-resistance to non-β-lactam antibiotics. Phenotypic detection of AmpC β-lactamase by the screening of cefoxitin-resistant isolates revealed that 43 (38.05%) isolates were considered positive for AmpC β-lactamase production. However, the PCR technique gives different results. In conclusion, the prevalence of ESBL and AmpC β-lactamase producing E. coli is rapidly increasing among clinical isolates of MDR, XDR, and possibly PDR E.coli.

Keywords: β-lactamase, Extended-Spectrum β-lactamases (ESBLs), AmpC β-Lactamases, Escherichia coli, Multidrug-Resistant (MDR), Extensively Drug-Resistant (XDR), Pandrug Resistant (PDR).

انتشار إنزيمات البيتا لاكتاميز ممتدة الطيف وإنزيمات الAmpC في العزلات السريرية لبكتريا Escherichia coli

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التخليص

تهدف الدراسة الحالية إلى التحقق من مدى انتشار إنزيمات البيتا لاكتاميز ممتدة الطيف في بكتريا Escherichia coli.

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The detection of ESBLs may be obscured by high AmpC negative results in phenotypic methods. Some methods, such as using clavulanic acid and sulbactam, do not efficiently inhibit ESBLs. Therefore, AmpC β-lactamase is considered one of the major β-lactam antibiotic resistance mechanisms in Gram-negative bacilli, including Escherichia coli. There are two important types of β-lactamases: the extended-spectrum β-lactamases (ESBLs) and AmpC, both of which confer resistance to extended-spectrum cephalosporins, which create serious therapeutic problems. Klebsiella spp. and Escherichia coli are the most commonly produced of these enzymes, although other Gram-negative bacteria can be producers. ESBLs are class A or D β-lactamases of Ambler that provide resistance to monobactams and third- and fourth-generation cephalosporins. Nevertheless, antibiotics like cephamycins, carbapenems, and β-lactamases inhibitors like clavulanic acid, sulbactam, and tazobactam inhibit ESBLs. Although that is the case, combined cephamycin and carbapenem resistance has been observed in ESBL-producing organisms. Resistance to additional types of antimicrobial drugs, such as tetracyclines, fluoroquinolones, aminoglycosides, and cotrimoxazole, is developing in healthcare settings. AmpC is a class C β-lactamase that, when produced in large amounts, provides resistance to a variety of β-lactam antibiotics, such as oxymino-cephalosporins and specific cephamsins, in addition to penicillins and monobactam. However, β-lactamase inhibitors like clavulanic acid and sulbactam do not efficiently inhibit AmpC β-lactamase. In some situations, the production of both plasmid-mediated AmpC and ESBLs may cause false-negative results in phenotypic methods for the detection of ESBLs (using clavulanic acid). As a result, AmpC co-production has complicated the detection of ESBL phenotypes. The detection of ESBLs may be obscured by high-level AmpC production. Furthermore, clavulanic acid may function as an inducer of high levels of AmpC, leading to false-negative ESBL testing. As a solution to this problem, tazobactam and sulbactam are recommended as inhibitors for ESBL testing because they are less likely to stimulate AmpC β-lactamases. Since
cefeipime (a fourth-generation cephalosporin) is unaffected by high levels of AmpC production, it can be the most reliable agent for the detection of ESBL production in the presence of AmpC β-lactamases [6]. The current study aims to investigate the production and prevalence of extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases enzymes in multiple drug-resistant E.coli isolates (MDR, XDR, and PDR), collected from different clinical specimens of hospitalized patients in Baghdad city.

2. Material and methods

2.1. Clinical isolates:
In a previous study, one-hindered and thirteen (113) clinical isolates of E.coli were collected from hospitalized patients in Baghdad [13]. The clinical specimens included urine, stool, blood, wound swabs, ear swabs, pus, abscesses, sputum, and body fluids (e.g., ascitic fluid, intrabdominal fluid, and CSF). The 113 E.coli isolates were identified using cultural and biochemical tests according to Bergey’s Manual [14]. Additionally, phenotypic identification of the isolates was genetically confirmed by targeting the uidA gene that codes for β-D-glucuronidase [15], [16].

2.2. Antimicrobial susceptibility test:
The antimicrobial susceptibility test was applied to all isolates that were identified as E.coli. The test was performed on Muller-Hinton agar (Oxoid, England) using the Kirby-Bauer disk diffusion method and according to the CLSI guidelines [17]. The test was performed using twenty (20) antimicrobial agents that fall within thirteen (13) different antimicrobial categories. The antimicrobial agents include: Piperacillin-tazobactam (100/10 μg), Ampicillin (10 μg), Piperacillin (100 μg), Cefotaxime (30 μg), Ceftazidime (30 μg), Ceftriaxone (30 μg), Cefepime (30 μg), Cefoxitin (30 μg), Imipenem (10 μg), Meropenem (10 μg), Aztreonam (30 μg), Gentamicin (10 μg), Amikacin (30 μg), Ciprofloxacin (5 μg), Levofloxacin (5 μg), Tetracycline (30 μg), Azithromycin (15 μg), Chloramphenicol (30 μg), Trimethoprim-sulfamethoxazole (1.25/23.75 μg), and Nitrofurantoin (300 μg). The antimicrobial disks were provided by Bioanalyse, Turkey.

2.3. Detection of Extended-Spectrum β-lactamases (ESBLs) by Double-Disc Synergy Test (DDST) methods:
the detection of ESBL production in AmpC β-lactamase co-producers, this test was modified from the original Double-Disc Synergy Test (DDST). Using a piperacillin-tazobactam (100/10μg) disc in place of amoxicillin-clavulanate (20/10μg) [6], [17]. The test was performed by placing a piperacillin-tazobactam (100/10μg) disc in the center of the Muller-Hinton agar plate inculcated with E.coli, then three discs of cephalosporins [Ceftazidime (30μg), Ceftriaxone (30μg), and Cefepime (30μg)] were placed at a distance of about 20 mm around the central disc [Piperacillin-tazobactam (100/10μg)]. The positive result (production of ESBLs) was indicated by the inhibition zone around any of the cephalosporin discs towards the piperacillin-tazobactam disc.

2.4. Screening for Cefoxitin Resistant E.coli (Detection of AmpC β-lactamases phenotypically)
The screening was performed according to Lorian [18]. Isolates with inhibition zones of less than 18 mm surrounding the cefoxitin disc (30 μg), were considered AmpC positive [19], [20].

2.5. Molecular Detection of AmpC (blaCMY) Gene
From each bacterial isolate, the genomic DNA was extracted using an ABIOpure™ Total DNA kit (ABIOpure, USA). Then the polymerase chain reaction was performed using the universal primers (the forward primer 5´-ATGATGAAAAATTACGTTATGC-3´ and the
reverse primer 5´-TTGCAGCTTTTCAAGAATGCGC-3´) for amplifying the \( \text{bla} \text{AmpC} (\text{blaCMY}) \) gene fragment with a 1,143 bp amplicon. Each 20 μl of the PCR reaction mixture contained 10μl of green master mix (Promega, USA), 1μM of both forward and reverse primers, 3 μl of the DNA template, and 5 μl of nuclease-free water (Promega, USA). The thermal cycler conditions were as follows: initial denaturation was performed at 95°C for 5 min, then 30 cycles of denaturation at 95°C for 30 sec, the annealing was at 56 °C for 30 sec, the extension was at 72°C for 30 sec, and the final extension was at 72°C for 7 min. Finally, the PCR amplification was confirmed using agarose gel electrophoresis with 1.5% agarose and 10mg/ml of ethidium bromide solution (Promega, USA). Electrical power was turned on at 100 v/mAmp for 75 minutes.

2.6. Statistical analysis:
The statistical analysis was carried out using GraphPad Prism version 5. For the purpose of comparing study samples, percentages were used. Chi-square was used in the comparison of categorical data during data analysis. Paired t-tests; and one-way ANOVA (Kruskal-Wallis test) were used to compare all selected data. All statistical tests were performed at a 5% significance level. Post-test p values are as follows: *p < 0.05; **p < 0.01; ***p < 0.001.

2.7. Ethical approval:
Ethical approval was obtained from the College of Science Research Ethics Committee at the University of Baghdad. Patients also filled out consent forms for specimen collection.

3. Results and discussion
3.1. Clinical isolates and antimicrobial susceptibility test:
The antimicrobial susceptibility test exhibited that out of 113 \( E. coli \) isolates, 111 (98.23%) were classified as multidrug-resistant (MDR). While only 2 (1.77%) isolates were susceptible to almost all antimicrobial agents (not classified as MDR), furthermore, the results showed that out of 113 isolates, 24 (21.24%) were classified as extensively drug-resistant (XDR), and only 2 isolates (1.77%) were classified as possibly pan-drug-resistant (PDR). All 113 isolates were subjected to the Double-Disc Synergy Test (DDST) for detection of ESBL production.

3.2. Production of Extended-Spectrum \( \beta \)-lactamases (ESBLs):
This test was accomplished by the Double-Disc Synergy Test (DDST) for detection of ESBLs in \( E. coli \) isolates using a piperacillin-tazobactam (100/10μg) disc in place of amoxicillin-clavulanate (20/10μg) to detect ESBLs in AmpC \( \beta \)-lactamases co-producers isolates. AmpC \( \beta \)-lactamases can mask the increase in zone diameter in DDST caused by ESBL producers since they are resistant to \( \beta \)-lactamase inhibitors such as clavulanic acid. It has been suggested and established that the most sensitive test for ESBL detection in isolates that co-produce AmpC \( \beta \)-lactamase is to modify DDST by using a combination of Piperacillin-Tazobactam and Cefepime, which is less affected by AmpC \( \beta \)-lactamases [6]. The results in Table 1 and Figure 1 showed that from a total of 113 \( E. coli \) isolates, 75 (66.37%) were ESBL-producing (positive for the ESBLs test), while 38 (33.63%) were not producing (negative for the ESBLs test) (Figure 2). These results indicated that the production of ESBLs among \( E. coli \) isolates was high with significant differences (P<0.02), which can explain the increase in antimicrobial resistance among \( E. coli \) isolates and the prevalence of multidrug-resistant (MDR) \( E. coli \) in the current study. Similar results were obtained from the study conducted by Kaur et al. [6], who found that 66.1% of tested isolates (\( E. coli \) in addition to other members of Enterobacteriaceae) were positive for the ESBL production test. The same study showed that 70.7% of \( E. coli \), in particular, were positive for ESBL production. Moreover, the current results were close to the results of Gupta et al. [10], who found that the percentage of ESBL-positive \( E. coli \) was 52.6% and that of ESBL-negative \( E. coli \) was 47.3%. Many other studies in the world have
demonstrated the high prevalence of ESBL production among E.coli strains [21], [22]. Nevertheless, the current results disagree with the study of Aabed et al. [23], who found that only 16.7% of E.coli isolates collected from urine samples were positive for the ESBL production test. Likewise, the results disagreed with the study of AL-Khazraji [24], who exhibited that 49.5% of E.coli isolates were positive for the production of ESBLs. The differences in the prevalence rates of ESBL production among bacterial isolates are very significant worldwide, can vary according to geographical areas, and fluctuate over time. This can be attributed to the ESBLs’ epidemiology, the variation in the size and type of tested samples collected from various geographical areas, and the approaches used for the detection of ESBLs [21], [25]. Since their initial description more than 20 years ago, ESBL-producing microbes have become a source of ever-growing concern. Numerous studies conducted in India have shown a prevalence incidence of 35%–85%. According to a Ugandan study, 62% of isolates produce ESBLs. Up to 32% of E. coli isolates and up to 58% of Klebsiella pneumoniae isolates in Latin America are ESBL-positive [5]. Table 1 showed that the distribution of ESBL production among 111 MDR and 24 XDR clinical isolates of E.coli was as follows: out of 111 MDR clinical isolates of E.coli, 64 (57.66%) isolates exhibited positive ESBL tests, while 47 (42.34%) isolates did not. Additionally, from a total of 24 XDR clinical isolates of E.coli, 11 (45.83%) isolates demonstrated positive ESBL tests, while 13 (54.17%) isolates showed negative ESBL tests, as shown in Figs. 3, and 4.

These results suggested that about half the rate of antimicrobial resistance in E.coli isolates was attributed to ESBL production, which emphasized the role of ESBL production in the antimicrobial resistance phenomenon. This result was similar to the result established by Subedi et al. [25], who found that 73.91% of MDR E. coli were ESBL producers. Additionally, Gupta et al. [10] stated that ESBL-producing isolates are significantly more multidrug-resistant than ESBL-negative isolates; consequently, the selection of antibiotics for therapy is limited. Figure 5 shows the distribution of ESBL production among 75 clinical isolates of E.coli according to the source of infections. The 75 isolates that showed positive production for ESBLs were obtained from different clinical specimens as follows: 43 (57.33%) from urine samples, 15 (20%) from stool, 7 (9.33%) from wound swabs, 4 (5.33%) from pus, and 2 (2.67%) from each of body fluids, blood, and sputum samples.

These results agreed with the results of Shashwati et al. [22], who found that most ESBL-producing isolates were collected from urine (52.28%), and also agreed with the results obtained by AL-Khazraji [24], who found that the highest percentage of ESBL production was obtained from urine (60.1%). Moreover, the results (Table 2) demonstrated that out of 75 ESBLs’ positive isolates, 39 (52%) were collected from females and 36 (48%) were obtained from males, so females were more frequent than males, with no significant differences (Figure 6). These results can be attributed to the fact that females’ samples were more frequent than males’ and related to the fact that UTI is more frequent in females than males, and more than half of the positive ESBL isolates were obtained from urine samples, as mentioned in the previous study [13]. These results agreed with the results obtained by AL-Khazraji [24], who found that the total number of ESBLs in females was higher than that in males. Likewise, a study by Azekhueme et al. [21] found that ESBL-producing bacteria in females were detected at the highest rate (57.5%) compared with their male counterparts (42.5%), but statistically, there is no significant difference between the two genders.

However, the current results disagree with Shashwati et al. [22], who found that the prevalence of ESBL producers was higher among males than females. The results (Table 3) showed that the distribution of ESBL production among 75 ESBLs’ positive clinical isolates of E.coli according to age groups was as follows: the highest production of ESBLs was obtained from the age group 41–60 years with 22 (29.33%) isolates from the total 75 ESBLs’ positive isolates,
followed by the age groups less than 20 years and more than 60 years with 18 (24%) isolates of each of them, and finally, the age group 20–40 years with 17 (22.67%) isolates. Nevertheless, the variances among the age groups were not statistically significant (Figure 7). Systemic infections caused by Enterobacteriaceae that produce ESBLs were linked to extremely adverse health consequences. ESBLs were initially identified in certain bacteria like \textit{E. coli} and \textit{Klebsiella} spp.; however, they have now expanded to additional genera, particularly \textit{Enterobacter} and \textit{Proteus} spp. Nowadays, ESBLs and AmpC Enterobacteriaceae co-producers are more frequently observed in various regions of the world [5].

\begin{table}
\centering
\caption{Distribution of ESBL production among clinical isolates of \textit{E. coli}}
\begin{tabular}{|l|c|c|c|c|}
\hline
 & Positive ESBLs & Negative ESBLs & Total & \textbf{P value} \\
\hline
\textit{E. coli} isolates & 75 (66.37\%) & 38 (33.63\%) & 113 (100\%) & 0.02 \\
\hline
MDR \textit{E. coli} & 64 (57.66\%) & 47 (42.34\%) & 111 (100\%) & 0.03 \\
\hline
XDR \textit{E. coli} & 13 (45.83\%) & 11 (54.17\%) & 24 (100\%) & 0.06 \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{Distribution of ESBL production among 75 clinical isolates of \textit{E. coli} according to the gender of patients}
\begin{tabular}{|l|c|c|c|}
\hline
 & Male & Female & \textbf{P value} \\
\hline
\textit{E. coli} isolates & 36 & 39 & \textit{P}\textgreater{}0.05 \\
\hline
48.00\% & 52.00\% & \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{Distribution of ESBL production among 75 clinical isolates of \textit{E. coli} according to age groups of patients}
\begin{tabular}{|l|c|c|c|c|}
\hline
 & < 20 years & 20–40 years & 41–60 years & \textit{> 60 years} \\
\hline
\textit{E. coli} isolates & 18 & 17 & 22 & 18 \\
\hline
24.00\% & 22.67\% & 29.33\% & 24.00\% & \textit{P}\textgreater{}0.05 \\
\hline
\end{tabular}
\end{table}

\begin{figure}
\centering
\caption{Production of ESBLs among 113 clinical isolates of \textit{E. coli}}
\end{figure}
Figure 2: Double-Disc Synergy Test (DDST) for detection of ESBLs in *E. coli* isolates. A and B: positive ESBLs test, C and D: negative ESBLs test

Figure 3: Distribution of ESBL production among 111 MDR clinical *E. coli* isolates

Figure 4: Distribution of ESBL production among 24 XDR clinical *E. coli* isolates

Figure 5: Distribution of ESBL production among 75 clinical isolates of *E. coli* according to the source of infections
3.3. Distribution of antimicrobial susceptibility among positive and negative ESBLs’ E.coli isolates:

The results of the present study showed that positive ESBL E.coli isolates were more drug-resistant than negative ESBL isolates (Figs. 8 and 9). The results found that the ESBL-producing E.coli isolates (positive ESBL-producing E.coli), exhibited a greater resistance rate to the β-lactam antibiotics (penicillins and cephalosporins), and this result was logical as these isolates produce ESBL enzymes that destroy β-lactam antibiotics. The results showed that the resistance rate to each of ampicillin, piperacillin, and cefotaxime was 100%; in addition, the resistance rates for both ceftazidime and ceftriaxone were 98.67%; and for cefepime it was 94.67%. On the other hand, the resistance rates for the β-lactams antibiotics (penicillins and cephalosporins) in the ESBL non-producing E.coli isolates (negative ESBL E.coli) were lower than the positive ESBL isolates. The resistance rates for each of ampicillin, piperacillin, cefotaxime, ceftazidime, ceftriaxone, and cefepime were 86.84%, 89.47%, 78.95%, 78.95%, 73.68%, and 65.79%, respectively. Moreover, the results showed that co-resistance to other groups of antibiotics (non-β-lactam antibiotics) like Aztreonam (80.00%), Ciprofloxacin (86.67%), and Nitrofurantoin (61.33%) was also higher with the ESBL producers (positive ESBLs E.coli).

However, in the cases of tetracycline, azithromycin, and trimethoprim-sulfamethoxazole, the rate of resistance was slightly higher in the negative ESBL isolates than in the positive ESBL isolates. The rates of resistance to tetracycline, azithromycin, and trimethoprim-sulfamethoxazole in the negative ESBL isolates were 81.58%, 86.84%, and 78.95%, respectively, whereas in the positive ESBL isolates they were 80.00%, 80.00%, and 74.67%, respectively. The high resistance rate in the negative ESBL isolates to these classes of antibiotics may be attributed to resistance mechanisms other than ESBL production, such as; AmpC production, metallo-β-lactamase production, efflux pumps, or other types of mechanisms.

The results of the current study agreed with those of Gupta et al. [10], who found that ESBL-positive isolates were more drug-resistant than ESBL-negative isolates. Another study by Subedi et al. [25] found that even though they show in vitro susceptibility, ESBL producers are naturally resistant to all cephalosporins. Additionally, ESBL production coexists with resistance to a number of other antibiotics. When the susceptibility patterns for ESBLs and non-ESBLs producers to non-β-lactam antibiotics were tested, a co-resistance to non-β-lactam antibiotics was noticed more with the ESBLs producers. For instance, the same study found a co-resistance to the fluoroquinolones.
(88.10–95.23%), gentamicin (73.81%), and co-trimoxazole (80.96%) [25]. Many other studies reported a co-resistance for non-β-lactam antibiotics among ESBL-producing isolates [6], [12], [22], [26]. Furthermore, the results of the current study indicated that the most effective antibiotics for positive ESBL isolates were meropenem, imipenem, and piperacillin-tazobactam, with sensitivity rates of 90.67%, 82.67%, and 78.67%, respectively. While the most effective antibiotics for negative ESBL isolates were gentamicin and meropenem, with sensitivity rates of 71.05% and 65.79%, respectively, similar results were observed in the study conducted by Subedi et al. [25], who found that 95.23% of ESBL-producing isolates exhibited susceptibility to piperacillin-tazobactam; in addition, 92.85% and 90.47% of these isolates showed susceptibility to imipenem and meropenem, respectively.

Also, the same study showed that 90.47% of ESBL-producing bacteria were sensitive to Nitrofurantoin and 80.9% were sensitive to Amikacin, which disagreed with the current study that found a different sensitivity rate among ESBL-producing isolates against Nitrofurantoin (5.33%) and Amikacin (44%). These differences can be attributed to the overuse and misuse of these two drugs in Iraq, especially for the treatment of UTIs, which encouraged an increase in the resistance rate against these two drugs. Likewise, Khan and Bari [26] found that 100% of ESBL-producing E. coli were sensitive to both imipenem and meropenem, plus 92% of isolates were sensitive to piperacillin-tazobactam. Additionally, Shashwati et al. [22] found that all ESBL producers’ bacteria were sensitive to imipenem, and most of them (80%) were sensitive to piperacillin-tazobactam, amikacin, and meropenem. Regarding the ESBL-negative strains, Gupta et al. [10] found that the isolates were sensitive to piperacillin-tazobactam, cefoperazone-sulbactam, carbapenems, and aminoglycosides.

The significant non-β-lactam antibiotic resistance of the strains that produce ESBLs increases the risk of treatment failure and reduces the therapeutic options for carbapenems. Therefore, the emergence of carbapenem resistance is a phenomenon of major concern for treating infections caused by multidrug-resistant bacteria. Although combinations of β-lactam/β-lactamase inhibitors have been recommended as a possible therapy for ESBL producers, these medications must be administered at high doses less frequently so that their serum and tissue levels are higher, with a consequently greater clinical success rate [25] [27]. The prevalence of ESBL-producing bacteria has been growing rapidly all over the world.

This condition is very concerning because ESBL producers have been found to show co-resistance to numerous groups of antibiotics, narrowing the treatment options [21]. The presence of ESBLs-producers in a person could result in elevated antibiotic resistance since the plasmid that contains the ESBLs enzymes also contains resistance genes for other classes of antibiotics (e.g., aminoglycoside, trimethoprim-sulfamethoxazole, and quinolones), consequently limiting the treatment options. The problem was complicated by the rapid spread of plasmid-mediated ESBL enzymes among different species of bacteria, which led to several nosocomial epidemics [6], [21], and [22]. Antimicrobial agents that are frequently used in our area, like trimethoprim-sulfamethoxazole, tetracycline, and ciprofloxacin, were found to be among the least effective against the ESBLs in this research. The excessive misuse and abuse of these cheap antimicrobial agents, which are easily accessible as over-the-counter (OTC) medications and can even be bought without a doctor's prescription, could be the reason for this problem. Accordingly, this creates challenges for the treatment of infections brought on by ESBL producers because these drugs are frequently administered as treatment options [21], [28].
3.4. Phenotypic and Molecular detection of AmpC β-lactamase

The results of phenotypic detection of AmpC β-lactamase by the screening of cefoxitin-resistant isolates revealed that out of 113 isolates, 43 (38.05%) showed an inhibition zone less than 18 mm surrounding the cefoxitin disk and were considered positive for AmpC β-lactamases production (Figure 10). This result was close to the result obtained by Kazemian et al. [29], who found that 29.2% of E.coli bacteria were ampC positive by the screening of cefoxitin-resistant disks. However, the results of the current study were higher than those of Bokaeian and Shayan [30], who found that 5% of E.coli isolates were resistant to cefoxitin (AmpC producers). Additionally, the current study found that of the total 43 isolates (potentially positive for AmpC β-lactamase by screening test), 23 (53%) were ESBL producers, while 20 (47%) were negative for ESBL production. Moreover, of these 43 isolates, 18 (42%) were classified as MDR, 23 (53%) were classified as XDR, and 2 (5%) were classified as PDR (Figure 11). This result showed that more than half of cefoxitin-resistant isolates were ESBL producers and classified as XDR; this percentage was higher than the result obtained from Rizi et al. [31], who found that 30% of cefoxitin-resistant bacteria simultaneously exhibited ESBL and 22% of isolates exhibited the MDR phenotype.

![Figure 8: Antimicrobial Susceptibility Test for 75 Positive ESBL E.coli Isolates](image1)

![Figure 9: Antimicrobial Susceptibility Test for 38 Negative ESBL E.coli Isolates](image2)
For molecular detection of AmpC β-lactamase genes, PCR for detection of the \( \text{bla}_{\text{AmpC}}(\text{blaCMY}) \) gene was applied to 25 \( \text{E.coli} \) isolates distributed as follows: 11 isolates were classified as MDR, 10 isolates were classified as XDR, 2 isolates were classified as possibly PDR, and another 2 were sensitive isolates (Table 4).

The results in Table 5 showed that out of 25 tested isolates, only 8 (32%) possessed the \( \text{bla}_{\text{AmpC}}(\text{blaCMY}) \) gene (Figure 12). All 8 isolates that possessed the \( \text{bla}_{\text{AmpC}}(\text{blaCMY}) \) gene were positive for screening with the cefoxitin test. Additionally, 2 of 8 isolates (25%) carried the \( \text{bla}_{\text{AmpC}}(\text{blaCMY}) \) gene, which is positive for both AmpC and ESBLs β-lactamase. While 6 of 8 isolates (75%) were positive for AmpC, but negative for ESBLs β-lactamase. Furthermore, the majority of isolates that possessed the \( \text{bla}_{\text{AmpC}}(\text{blaCMY}) \) gene [6 of 8 (75%)] were classified as XDR and possibly PDR. This result disagreed with other studies that used PCR techniques for the detection of AmpC β-lactamase genes [19], [30], [29], and [31]. The differences in the results of PCR among the current and other studies can be attributed to many reasons, such as: (1) The production of AmpC β-lactamases can be controlled by many families of genes, e.g., \( \text{bla}_{\text{ACC}}, \text{bla}_{\text{DHA}}, \text{bla}_{\text{EBC}}, \text{bla}_{\text{FOX}}, \text{bla}_{\text{MOX}}, \text{bla}_{\text{CMY}}, \) and \( \text{bla}_{\text{CIT}} \). (2) Additionally, the differences among studies can be attributed to the differences in the size and types of samples, their sources of infection, and the geographic area, in addition to the period of study. For these reasons, it is hard to make a comparison of AmpC β-lactamases prevalence among studies.

**Figure 10**: Phenotypic detection of AmpC β-lactamase by screening for Cefoxitin-resistant among 113 clinical isolates of \( \text{E.coli} \).

**Figure 11**: Distribution of MDR, XDR, and PDR \( \text{E.coli} \) isolates among 43 isolates that were positive for AmpC β-lactamase by a screening test for Cefoxitin-resistant

**Figure 12**: Gel electrophoresis of \( \text{E.coli} \) isolates for amplification of \( \text{bla}_{\text{AmpC}}(\text{blaCMY-2}) \) gene on 1.5% agarose stained with Ethidium Bromide, electrical power was turned on at 100v/mAmp for 75min. M: 100bp ladder marker; product size 1143bp; lanes: 33, 75, 79, 10, 23, 87, 108, 109: Positive; NC: Negative Control.
The results of the present study indicated that ESBL and AmpC co-producers can emerge among Iraqi clinical isolates of E. coli. This finding agreed with the findings from previous research [31, 32], which found that some ESBL producer isolates may also be AmpC producers and may contain multiple AmpC cluster genes. Numerous other studies have raised a significant alarm regarding the treatment and control of infections brought on by ESBL and AmpC co-producer bacteria [19], [30], and [29].

Table 4: Results of 25 E. coli isolates for phenotypic AmpC β-lactamase production, ESBL production, and detection of blaAmpC (blaCMY-2) gene by PCR technique.

<table>
<thead>
<tr>
<th>E. coli Isolates</th>
<th>Phenotypic detection of AmpC β-lactamase by screening for Cefoxitin resistant ESBLs production</th>
<th>Detection of the blaAmpC (blaCMY) gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>XDR R (Positive)</td>
<td>Positive</td>
</tr>
<tr>
<td>10</td>
<td>XDR R (Positive)</td>
<td>Negative</td>
</tr>
<tr>
<td>12</td>
<td>S S (Negative)</td>
<td>Negative</td>
</tr>
<tr>
<td>17</td>
<td>MDR S (Negative)</td>
<td>Positive</td>
</tr>
<tr>
<td>23</td>
<td>MDR R (Positive)</td>
<td>Negative</td>
</tr>
<tr>
<td>27</td>
<td>S S (Negative)</td>
<td>Negative</td>
</tr>
<tr>
<td>31</td>
<td>MDR R (Positive)</td>
<td>Positive</td>
</tr>
<tr>
<td>33</td>
<td>XDR R (Positive)</td>
<td>Negative</td>
</tr>
<tr>
<td>36</td>
<td>MDR S (Negative)</td>
<td>Negative</td>
</tr>
<tr>
<td>49</td>
<td>XDR R (Positive)</td>
<td>Positive</td>
</tr>
<tr>
<td>51</td>
<td>MDR S (Negative)</td>
<td>Positive</td>
</tr>
<tr>
<td>56</td>
<td>XDR R (Positive)</td>
<td>Positive</td>
</tr>
<tr>
<td>60</td>
<td>XDR S (Negative)</td>
<td>Positive</td>
</tr>
<tr>
<td>61</td>
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</tr>
<tr>
<td>62</td>
<td>MDR R (Positive)</td>
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</tr>
<tr>
<td>66</td>
<td>XDR R (Positive)</td>
<td>Positive</td>
</tr>
<tr>
<td>67</td>
<td>PDR R (Positive)</td>
<td>Positive</td>
</tr>
<tr>
<td>75</td>
<td>XDR R (Positive)</td>
<td>Positive</td>
</tr>
<tr>
<td>79</td>
<td>PDR R (Positive)</td>
<td>Positive</td>
</tr>
<tr>
<td>83</td>
<td>MDR S (Negative)</td>
<td>Positive</td>
</tr>
<tr>
<td>87</td>
<td>MDR R (Positive)</td>
<td>Negative</td>
</tr>
<tr>
<td>102</td>
<td>MDR S (Negative)</td>
<td>Positive</td>
</tr>
<tr>
<td>108</td>
<td>XDR R (positive)</td>
<td>Positive</td>
</tr>
<tr>
<td>109</td>
<td>XDR R (positive)</td>
<td>Negative</td>
</tr>
<tr>
<td>111</td>
<td>MDR R (positive)</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Table 5: Characterization of 8 E. coli isolates that carried blaAmpC (blaCMY) gene detected by PCR

<table>
<thead>
<tr>
<th>E. coli isolates carried blaAmpC (blaCMY) gene (%)</th>
<th>E. coli isolates carried blaAmpC (blaCMY) gene and gave positive result for phenotypic screening of Cefoxitin resistant (%)</th>
<th>E. coli isolates carried blaAmpC (blaCMY) gene and positive for both AmpC and ESBLs β-lactamase (%)</th>
<th>E. coli isolates carried blaAmpC (blaCMY) gene and positive for AmpC, but negative for ESBLs β-lactamase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 (100%)</td>
<td>8 (100%)</td>
<td>2 (25%)</td>
<td>6 (75%)</td>
</tr>
</tbody>
</table>

The current study showed that AmpC screening and molecular tests produced different results, and this agreed with the study of Kazemian et al. [29], who stated that a high rate of false-negative results was reported by phenotypic detection methods for AmpC. Moreover, other studies demonstrated false-positive results by phenotypic detection methods of AmpC production [19], [30], and [31]. Cefoxitin resistance is used as a marker for the detection of AmpC-producers based on the CLSI criteria, but numerous studies, including the current one, have shown that not all cefoxitin-resistant isolates produce AmpC β-lactamases (false-positive results). The following can be used to explain this phenomenon: Firstly, there are other
enzymatic mechanisms for cefoxitin resistance besides AmpC β-lactamase production, such as extended-spectrum β-lactamases (ESBLs) and Metallo β-lactamases (MBL), as well as nonenzymatic mechanisms like porin channel mutation. Secondly, AmpC β-lactamase production can be controlled by many families of genes. Thirdly, phenotypic tests cannot distinguish between positive results due to chromosomally-mediated AmpC β-lactamases and those due to plasmid-mediated AmpC genes, additionally, mutations in the promoter and/or attenuator sections of the chromosomal AmpC gene can cause overexpression of the gene, leading to the cefoxitin-resistant phenotype in E.coli. Finally, cefoxitin is a substrate for an active efflux pump in some isolates [30], [31].

Significant clinical treatment failures with cephalosporins can be observed due to the high level of AmpC production. The prevalence of AmpC β-lactamases is not well understood, and this may be because of a lack of accurate detection procedures in medical laboratories. The elevated prevalence of AmpC β-lactamases bacteria could be explained; as samples were obtained from inpatients and patients admitted to the intensive care unit, it was reasonable to assume that they had previously received cephalosporin therapy, either based on clinical judgment or by the hospital's antibiotic policy. As a consequence, this can generate selective pressure, which is one of the contributing factors raising the prevalence of AmpC production [19]. Detecting ampC-producers may be clinically important not only because of their higher cephalosporin resistance but also because carbapenem resistance can develop through additional mutations, resulting in reduced porin expression [19].

Briefly, ESBL and AmpC co-producers can arise among Iraqi clinical isolates of E.coli. Additionally, false positive or negative results encountered the phenotypic detection methods of AmpC production. Thus, the most reliable method for detecting AmpC β-lactamase is PCR. However, in some cases, false-negative results can occur with the PCR technique, and this could be accounted for by the fact that while the genes might be detected by PCR, they may not be efficiently expressed phenotypically [19].

Conclusion
In conclusion, the prevalence of ESBL and ampC β-lactamase producing E. coli is rapidly increasing in our country and among clinical isolates of MDR, XDR, and possibly PDR E.coli. This is due to the fact that the drug regulatory authority and health care commission play a minor or insignificant role in the rational use of antibiotics, the rules and regulations governing antibiotic use are poorly implemented, and there is a rise in quackery among medical professionals. A precise and accurate phenotypic test is required for detecting AmpC β-lactamases and distinguishing between AmpC and ESBL producers. Clinicians and healthcare systems need to be completely educated about ESBL and AmpC producers’ bacteria, it seems. Similarly, ESBL and AmpC production observation is recommended to prevent treatment failure and ensure effective infection control in Iraq.

References


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